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Biofilm formation of *Tenacibaculum maritimum*, a fish pathogenic bacteria, to evaluate the antimicrobial activity of fish skin mucus

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Biofilms, defined as aggregates of microorganisms embedded in a self-produced matrix of extracellular polymeric substances (EPS), are formed by most bacteria in both natural and pathogenic ecosystems. In aquaculture, biofilms pose a dual challenge: they confer recalcitrance to antimicrobials treatments and contribute to persistent infections by forming on facility surfaces such as tanks, nets, cages, and equipment. *Tenacibaculum maritimum*, the causative agent of tenacibaculosis, is responsible for significant economic losses in fish farming. Although the antibacterial activity of fish skin mucus against this pathogen has been evaluated *in vitro*, its effects on *T. maritimum* biofilms have not yet been determined. In this study, we provide a simple methodology for the *in vitro* formation and quantification of *T. maritimum* biofilms to monitor antibacterial properties of different compounds or substances, such as fish skin mucus. For this purpose, biofilm formation was assessed under varying culture volumes (200, 300, and 400 μ L) and incubation times (24, 48, and 72 hours) in 48-well microplates. Then, the effects of gilthead seabream (*Sparus aurata*) skin mucus were evaluated on planktonic growth, biofilm formation, and biofilm dispersion, measuring both biomass and metabolic activity. Based on the tested volumes and incubation times, the optimal condition for biofilm formation was defined as 24 hours in MB at 25 °C using 200 μ L culture volume. These conditions supported the development of a biofilm ($OD_{570} > 1.5$ after crystal violet staining) while conserving time and mucus. Seabream mucus significantly impaired *T. maritimum* planktonic growth and biofilm formation in a concentration-dependent manner. Non-diluted mucus completely inhibited planktonic growth and biofilm metabolic activity, and reduced biofilm biomass by $81.16 \pm 2.54\%$. In contrast, its effect on mature biofilms was limited, with reductions of approximately 50% in metabolic activity and 40% in biomass. This study provides a platform to assess how different fish culture conditions affect the host's susceptibility to *T. maritimum* infections, which is crucial for preventing economic losses in fish farming. Additionally, it opens the door to studies analyzing the components of fish skin mucus responsible for its antibacterial activity, aiming to develop novel therapeutic compounds for targeting biofilms formed by this pathogen.

KEYWORDS

Tenacibaculum maritimum, fish skin mucus, *Sparus aurata*, antibacterial, antibiofilm

1 Introduction

Upon attached to a solid surface, numerous bacteria initiate a complex process of coordinate differentiation and production of a protective extracellular matrix that will lead to the formation of a biofilm (Manning, 2003). Biofilms can be defined as aggregates of microorganisms embedded in a self-produced matrix of extracellular polymeric substances (EPS) that adhere to each other and/or to a surface, where cells differ from their planktonic form in terms of growth rate and gene expression (Rather et al., 2021c). These structures are produced by the vast majority of bacteria in most natural and pathogenic ecosystems (Stoodley et al., 2002). In clinical and veterinary microbiology, the presence of biofilms supposes a great challenge since elicit recalcitrance to antimicrobial treatments (Flemming et al., 2016; Koo et al., 2017). In aquaculture, biofilms pose a significant challenge—not only because they make treating infected fish more difficult, but also because they form on tank surfaces, nets, cages, equipment, and culture sediments. These biofilms serve as reservoirs for pathogens, increasing the risk of disease outbreaks (Acosta et al., 2021). Besides, biofilms also represent a major concern in postharvest processes in the fish and seafood processing industries (Rather et al., 2021a). Different human pathogenic bacterial species have been identified in fish and seafood, and the biofilms formed on the skin of the animals or the transport boxes may contaminate the surfaces of the processing plants (Rajkowski, 2009).

Tenacibaculum maritimum (previously *Flexibacter maritimum*) is a Gram-negative filamentous bacteria that accounts for the majority of tenacibaculosis cases in marine fish species (Fernández-Álvarez and Santos, 2018; Mabrok et al., 2023). This disease affects mainly external tissues, causing gross lesions on the body surface, including ulcerative skin lesions, tissue necrosis, fin and tail rots, mouth erosion, and gill and eye necrosis (Avendaño-Herrera et al., 2006b). Early-stage ulcerations can rapidly evolve into severe tissue damage or necrosis and subsequently into septicemia (Bernardet et al., 1994; Mabrok et al., 2023). Since the first description of this disease in red seabream and gilthead fry cultures in Japan (Masumura and Wakabayashi, 1977), this bacterium has been identified and isolated in numerous outbreaks affecting both food and ornamental fish cultures worldwide. Its ability to grow in temperatures ranging from 15°C to 34°C, coupled to its extensive broad host range, explains its extensive geographical distribution and the (re)-emergence of this pathogen (Mabrok et al., 2023). Significant economic losses occur due to the high mortality rates and the external lesions that diminish the economic value of the fish (López et al., 2009; Lopez et al., 2022; Masumura and Wakabayashi, 1977; Handler et al., 1997).

Although the pathogenic mechanisms of *Tenacibaculum maritimum* are not yet fully understood, evidence shows that *Tenacibaculum* species can be found in tidal flats (Choi et al., 2006; Li et al., 2022; Shang et al., 2021) and survive for extended periods in seawater microcosms (Avendaño-Herrera et al., 2006a), indicating that sediments may serve as natural reservoirs for these bacteria (Mabrok et al., 2023) and water being considered an important route of infection. Bacterial cells present in the water can adhere to and colonize the surfaces of fish, particularly targeting areas such as the skin and mucus layer. Fish rely on a continuous production and

secretion of mucus on their skin that acts as the first line of defense against a variety of environmental conditions or stressors, including bacterial infections (Fernández-Alacid et al., 2018). Skin mucus is composed of a matrix of glycoproteins (mucins) that contains a wide range of molecules, some of them involved in protecting fish against pathogens. The presence of antimicrobial compounds in skin mucus, including immunoglobulins, lysozyme, lectins, secondary metabolites, and antimicrobial peptides, is well documented (Esteban, 2012). Consequently, fish mucus is a potential source of new therapeutic agents against bacterial infections (Díaz-Puertas et al., 2023; Tiralongo et al., 2020). Lacking flagella, pili, and fimbriae, *T. maritimum* adhesion is mediated by adhesins, exopolysaccharides and proteins displaying lectin or sugar-binding motifs (Pérez-Pascual et al., 2017). Binding to the carbohydrate residues in skin mucus may be the first step in *T. maritimum* infection, and its progress will depend on its ability to evade the immunological strategies and the antimicrobial compounds in skin mucus (Mabrok et al., 2023). It is well known that environmental and culture conditions can alter fish immunity (Bowden, 2008; Corbel, 1975; Magnadottir, 2010; Watts et al., 2001). The antibacterial activity of skin mucus suffers changes in front of different challenges, such as dietary conditions (Fernández-Alacid et al., 2021, 2022; Firmino et al., 2021; Gisbert et al., 2021; Sanahuja et al., 2023a), stress situations (Herrera et al., 2020; Tacchi et al., 2015), anaesthetic treatments (Soltanian et al., 2018), and pathogen challenges (Guardiola et al., 2019; Kumari et al., 2019). Hence, *T. maritimum* cells adhering to the skin mucus of carrier fish may initiate an infection process when environmental conditions become favorable or in immunosuppression conditions. Despite the described adherence of *T. maritimum* to different surfaces (Burchard et al., 1990; Magariños et al., 1995; Sorongon et al., 1991) and its ability to form cell aggregates when incubated in both static and slight shaking conditions (Mabrok et al., 2016; Wakabayashi et al., 1986; Yamamoto et al., 2010), scarce works studying *T. maritimum* biofilms are available (Escribano et al., 2023; Levipan et al., 2019). In addition, biofilms have not been usually included in studies assessing the antibacterial activity of fish skin mucus against this pathogen, although biofilm formation is presumably a necessary step in the establishment of the infection by *Tenacibaculum* (Avendaño-Herrera et al., 2006b; Levipan et al., 2019).

In this context, the present study aims to establish a simple and reproducible protocol for the formation and quantification of *T. maritimum* biofilms that will allow a more realistic approximation when studying the antibacterial properties of different compounds against this pathogen. For this purpose, a first attempt to elucidate the effects of gilthead seabream (*Sparus aurata*) skin mucus on *T. maritimum* planktonic and biofilm growth was included.

2 Materials and methods

2.1 Animal conditions, skin mucus sampling and processing

Gilthead seabream juveniles were obtained from a local fish farm and maintained in 400 L tanks (3 kg biomass·m⁻³) at the University of Barcelona indoor facilities at 22°C under a 12 h light: 12 h dark

photoperiod. Fish were fed daily with a commercial standard diet (DIBAQ, Optimus) at a 3% body weight·day⁻¹ rate. After one month of acclimation, skin mucus was sampled.

Seabream individuals (body weight, 172 ± 5 g) from six different tanks were caught and anesthetized (100 mg/L tricaine methanesulfonate, MS-222, Sigma-Aldrich). Then, skin mucus was collected following a previously described method (Fernández-Alacid et al., 2018). Briefly, mucus was gently removed using sterile glass slides, from the skin above the lateral line of the fish in a front-to-caudal direction and collected in sterile microtube. Ventral zones were excluded to avoid fecal and urogenital contamination. For each tank, the skin mucus of nine fish was randomly collected. The skin mucus samples were homogenized with a sterile Teflon sticker and centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatants (soluble fractions of mucus) were retrieved and stored at -80°C. An amount of approximately 250–300 mg of crude mucus per fish could be obtained, and the soluble fraction represents approximately 60–80% of this weight. Thus, the total amount of soluble mucus obtained per fish ranged from 150 mg to 240 mg, corresponding approximately to 0.15–0.24 mL of mucus per fish. For the experiments conducted in this work, a total of 5.1 mL of soluble mucus per experiment (inhibition of planktonic growth, biofilm formation and dispersion) is required.

To ensure consistency across all assays, a pooled sample was prepared mixing the soluble fraction of the mucus of all the individuals sampled. This pooled sample was divided into different aliquots that were stored at -80°C until use. Also, its protein concentration was determined by the Bradford method (Bradford, 1976) using a bovine serum albumin (BSA, Sigma) standard curve. The Optical Density (OD) was determined at λ=596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain).

The experimental procedures in this study complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), as well as with the Spanish regulation (Law 32/2007 and RD 53/2013). The study was approved by the Ethics Committee of the University of Barcelona for the Use of Laboratory Animals and the Generalitat de Catalunya (DAAM 9383).

2.2 *Tenacibaculum maritimum* strain and growth conditions

Tenacibaculum maritimum CECT 4276 strain (Wakabayashi et al., 1986) from the Spanish Type Culture Collection (CECT, University of Valencia, Spain) was used. Following the CECT recommendations, *T. maritimum* was stored at -80°C in 20% glycerol and routinely streaked on Marine Agar (MA, BD Difco™) plates and incubated at 30°C for 48 h. Marine Broth (MB, BD Difco™) was used for liquid cultures, and was prepared at different concentrations: 1x, 2x and 3x.

2.3 Evaluation of skin mucus antibacterial activity on planktonic cultures

Overnight cultures (16 h, 25°C, shaking conditions) were used to inoculate 20 mL of fresh medium (MB) at an initial cell density of

~ 1x10⁷ CFU·mL⁻¹, which corresponds to an OD₆₀₀ of 0.04, and incubated in the same conditions until reaching exponential growth phase (~ 7x10⁷ CFU·mL⁻¹, which corresponds to an OD₆₀₀ of 0.1). The assay of antibacterial activity of skin mucus was performed by monitoring the absorbance of bacterial cultures grown in flat-bottomed 96-well plates (Biolite Thermo Scientific) as previously described (Sanahuja et al., 2019). Aliquots of 50 µL of cultured bacteria were mixed with 50 µL of concentrated fresh medium (MB 3x) and 100 µL of skin mucus sample at 9.66 mg prot·mL⁻¹ (no diluted mucus), 4.83 mg prot·mL⁻¹ (1:2 diluted mucus), and 1.93 mg prot·mL⁻¹ (1:5 diluted mucus). Several controls were included in the plate: untreated control, where 50 µL of the same cultured bacteria were mixed with 150 µL of fresh medium (MB 1x); negative control, where 100 µL of the skin mucus sample dilutions used were incubated with 100 µL of medium (MB 2x); and media negative control, with 200 µL of MB (1x) were incubated. Triplicates were used for statistical purposes.

Bacterial growth was measured by absorbance at λ=400 nm every 30 min for 14 h at 25°C with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). A 30-second shaking was performed every 2 minutes.

Bacterial growth inhibition was calculated at even hours of culture, as:

% inhibition =

$$\left(\frac{OD_{400} \text{ of (sample - negative control)}}{OD_{400} \text{ of (untreated control - media negative control)}} - 1 \right) \times 100$$

2.4 Biofilm formation assay setup

Before assessing skin mucus effects on *T. maritimum* biofilm, the settlement of optimal conditions for *in vitro* biofilm formation was needed. These conditions were decided based on being less time-consuming and using the smallest amount of mucus (limited component). Therefore, an experiment evaluating biofilm formation in flat-bottomed 48-well-polystyrene plate at 25°C, using different inoculum volumes (200, 300, and 400 µL) and incubation times (24, 48, and 72 hours) was designed.

T. maritimum overnight culture was diluted with fresh medium (MB) to ~ 4.6x10⁶ CFU·mL⁻¹ (which correspond to an OD₆₀₀ of 0.02) to use as the inoculum. The different inoculum volumes used were placed (sextuplicate) in three 48-well-polystyrene plates. The same volumes of MB were used as negative controls. The plates, placed inside plastic bags with wet cellulose paper to maintain the proper humidity levels, were incubated statically at 25°C for 24, 48, and 72 h. After the incubation time, all liquid culture was removed from the plate and half of the wells were gently washed twice with distilled water. Next, all the wells were stained with Cristal Violet (CV) (see below). The negative control values were subtracted from the experimental values.

Once the optimal conditions for the biofilm formation were settled (24 h, 25°C and 200 µL inoculum), the effect of the presence of skin mucus in biofilm formation and biofilm dispersal assays were performed, by adapting the previously described method (Paytubi et al., 2017).

2.4.1 Inhibition of biofilm -formation assay

An overnight culture of *T. maritimum* diluted to $OD_{600} = 0.04$ ($\sim 1 \cdot 10^7$ CFU·mL⁻¹) in MB (2x) was used as inoculum. 100 μ L of bacterial suspension were incubated with 100 μ L of mucus at 9.66 mg prot·mL⁻¹ (no diluted mucus), 4.83 mg prot·mL⁻¹ (1:2 diluted mucus) or 1.93 mg prot·mL⁻¹ (1:5 diluted mucus) in triplicate. Several controls were included: untreated control (100 μ L of bacterial suspension were incubated with 100 μ L sterile MilliQ water); negative control (100 μ L of MB 2x were incubated with 100 μ L of mucus sample); and media negative controls (100 μ L of MB 2x were incubated with 100 μ L of sterile MilliQ water), in triplicate as well. The same bacterial suspension volume was used to validate the procedure with a gradient of ampicillin (6.25 μ g·mL⁻¹ to 0.049 μ g·mL⁻¹ in a 1/2 serial dilution). Two replicas of the flat-bottomed 48-well plates were statically incubated at 25°C for 24 hours. After the incubation, the quantitative measurement of the biofilm biomass was determined by CV staining. For quantitative monitoring of living cells within the biofilm, resazurin (RZN) staining was performed.

2.4.2 Biofilm dispersal assay

Mature biofilms of *T. maritimum* were obtained as described in the biofilm formation assay setup. After the incubation, planktonic cells were removed and 100 μ L of fresh media (MB 2x) were loaded to each well. 100 μ L of mucus, sterile water, and antibiotic gradient concentrations were added as described for the biofilm-formation assay. Plates were incubated in the same conditions for another 24 hours. Biofilm biomass and living cells were determined by CV and RZN staining.

2.4.3 Crystal Violet staining

Crystal Violet (CV) is a protein-dye that binds to negatively charged molecules, such as the peptidoglycan and the extracellular matrix (Petrachi et al., 2017). Hence, its use to quantify biofilm biomass in microtiter-plates is widely established (Stepanović et al., 2000). CV staining protocol was adapted from Paytubi et al. (2017), with some modifications. After removing planktonic cells from the plates, no washes with distilled water were performed (except for the assay used to establish the optimal biofilm forming conditions). Biofilms were fixed by heating at 80°C for 30 minutes. Next, staining was performed by adding CV (1% w/v in MilliQ water) to the each well and incubating the plates for 15 minutes at room temperature. The CV volume added to each well was the double of the final volume used in the assays to ensure that all biofilm biomass was stained (400, 600, or 800 μ L/well in the assay for the establishment of optimal conditions and 400 μ L/well for biofilm formation and elimination assays). Then CV solution was discarded, wells were washed with distilled water and plates were air-dried. Finally, CV was solubilized by adding acetic acid 30% (v/v) (200, 300, or 400 μ L/well in the assay for the establishment of procedural conditions and 200 μ L/well in the biofilm inhibition formation and dispersion assays). Biofilm biomass was determined by measuring the OD_{570} of the resulting solution in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain), after subtracting OD_{570} of the media negative controls.

2.4.4 Resazurin assay

Resazurin (RZN) is a blue, water-soluble, and non-fluorescent dye that, when reduced by electron transfer reactions of cell respiration, turns into a pink, fluorescent product (resorufin) easily measured by fluorescence (O'Brien et al., 2000). Therefore, resazurin can be used to monitor biofilm cell viability or metabolic activity (Mariscal et al., 2009). After removing of planktonic cells from the plates, no washes with distilled water were performed and 200 μ L of fresh culture medium (MB 1x) and 50 μ L of RZN solution (resazurin sodium salt (Sigma) at 0.02% w/v in MilliQ water and sterilized by filtration) were added to each well. Plates were statically incubated in the darkness at 37°C for 20–45 minutes. Fluorescence was measured (excitation 570 nm, emission 615 nm) with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain).

2.4.5 Biofilm inhibition analysis

Biofilm inhibition by skin mucus was evaluated integrating biofilm biomass (CV) and biofilm metabolic activity (RZN) inhibitions. For this purpose, the different inhibitions indexes were calculated with the following equations:

Biofilm biomass inhibition

% of inhibition

$$= \left(\frac{OD_{570} \text{ of (sample - media negative control)}}{OD_{570} \text{ of (untreated control - negative control)}} - 1 \right) \times 100$$

Biofilm viability inhibition

% of inhibition

$$= \left(\frac{\text{Fluorescence of (sample - media negative control)}}{\text{Fluorescence of (untreated control - negative control)}} - 1 \right) \times 100$$

2.5 Statistical analysis

Statistical analysis was performed using one-way ANOVA test of SPSS software Version 22.0 (IBM Corp, Armonk, NY, USA), followed by *post-hoc* Bonferroni's test (if equal variances were assumed by Levene's test) or Dunnett's test (if variances among groups were unbalanced). Differences between mucus dilutions were considered statistically significant at $p < 0.05$.

3 Results

3.1 Evaluation of skin mucus antibacterial activity against *T. maritimum*

The antibacterial activity of seabream skin mucus was tested on *T. maritimum* planktonic growth. The results obtained (Figure 1) showed that gilthead seabream skin mucus has a high antibacterial activity against this pathogen in a concentration-dependent manner. Non-diluted mucus (9.66 mg of protein·mL⁻¹)

completely inhibited *T. maritimum* growth, whereas 1:2-diluted mucus initially caused high inhibition, but the antimicrobial effect was reduced during the later hours of culture. On the other hand, a dilution of the mucus to 1.93 mg of protein·mL⁻¹ resulted in an almost complete loss of antibacterial activity against the pathogen.

3.2 Experimental setup conditions for *T. maritimum* biofilm formation

Biofilm formation assays were initially conducted in 96-well polystyrene plates, however, the data yielded was characterized by high variability and limited reproducibility. Furthermore, visual inspection of *T. maritimum* biofilms using crystal violet (CV) staining revealed notably irregular morphologies (data not shown). Due to the apparent weak adherence of the biofilm to the surface of the microplate wells, the *T. maritimum* biofilms could easily be disrupted by mechanical force and subsequently removed during washing steps (see [Supplementary Material](#)). The following biofilm assays were performed using 48-well plates. In addition to improve biofilm visualization, using 48-well plates offers several advantages over 96-well plates. The increased well size and culture volume in 48-well plates resulted in more consistent and reproducible biofilm formation data, as previously described ([Gomes and Mergulhão, 2021](#)). We evaluated different culture volumes and incubation periods, as well as non-washing or washing steps before fixing and CV staining, to determine the optimal experimental conditions for *T. maritimum* biofilm formation ([Figure 2](#)).

T. maritimum produce a visible thick biofilm in all the conditions tested. The biofilm formed was almost completely lost

during the two washing steps, as shown in [Figure 2A](#). The biofilm loss during washing was not proportional to the preexisting biofilm formed and was highly variable among replicas. These results suggested that the differences observed for washed biofilms were related to manipulation differences during the washing steps.

Regarding the non-washed biofilms, the highest biofilm biomass was achieved when the higher culture volumes (300 and 400 µL) were used, at both 48 and 72 hours of incubation. After 24h of incubation, the highest biofilm biomass was achieved using 200 µL of bacterial inoculum ([Figure 2](#)).

Having in consideration that fish skin mucus volume could be a limited component, it was considered that the biofilm biomass achieved after 24 hours with a 200 µL inoculum was suitable to study the putative effects of mucus on *T. maritimum* biofilm formation. These conditions allowed saving both incubation time and most importantly fish skin mucus volume. Considering all these results, the conditions for the biofilm assays were settled as follows: 200 µL culture volume by well using 48-wells microplates, 24 h of incubation at 25°C, and no washing steps before biofilms fixation. However, it subsequently implied detecting some non-attached sedimented cells as if they were part of the biofilm structure, a limitation that should be taken into account when analyzing the results.

3.3 Skin mucus antimicrobial effect on *T. maritimum* biofilm formation

The effect of the skin mucus on the inhibition of biofilm formation and the dispersion of previously formed biofilm was evaluated by measuring the biofilm biomass and the cell viability of

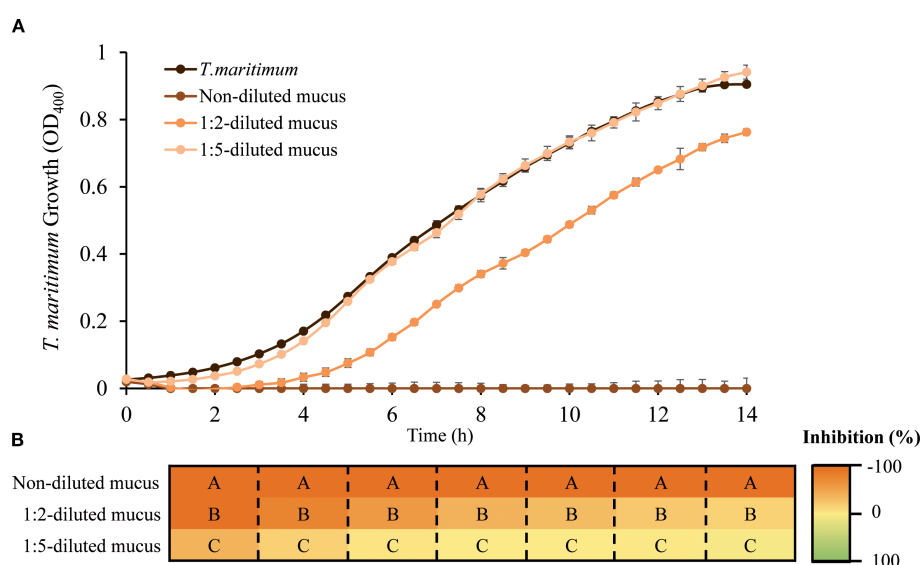


FIGURE 1

Skin mucus antibacterial activity against planktonic cultures of *T. maritimum*. (A) Growth of *T. maritimum* incubated without or with different dilutions of gilthead seabream skin mucus. Three different concentrations of mucus were used, corresponding to 9.66, 4.83 and 1.93 mg protein/mL (non-diluted, 1:2-diluted mucus and 1:5-diluted mucus respectively). Values are expressed as mean $\pm$ SEM (Standard Error of the Mean).

(B) Significant differences in growth inhibition caused at even hours of culture between mucus dilutions are indicated by capital letters (one-way ANOVA, $p < 0.05$).

biofilm cells and comparing the results with those obtained in the absence of skin mucus (Figure 3). A gradient of ampicillin concentrations was used to validate the results (Figure 3A).

The antimicrobial effect of the fish skin mucus on the biofilm formation shows similar results to those obtained when the effect of skin mucus was assayed in planktonic cells (Figure 3B). Non-diluted mucus strongly inhibited biofilm formation: a reduction of $81.16 \pm 2.54\%$ in biofilm biomass was observed and, remarkably, no metabolic activity was detected. The inhibitory effect decreased when 1:2-diluted mucus was used ($-34.90 \pm 4.26\%$ and $-19.24 \pm 1.90\%$ of reduction in biofilm biomass and cell viability respectively).

Undiluted gilthead seabream skin mucus demonstrated significant effects on mature biofilms, promoting substantial biofilm biomass dispersion ($-38.53 \pm 0.29\%$) and reducing the metabolic activity by approximately 50% (Figure 3B). In contrast,

diluted mucus appears not to affect biofilm biomass and viability in the biofilm dispersion assay.

There is a clear correlation between the quantity of skin mucus used in the assays and the effects caused in biofilm formation and dispersion of mature biofilm.

4 Discussion

Mucus plays an essential role in protecting fish against pathogens, not only by preventing bacterial contact with the underlying tissue (skin, gills, and intestine) but also by serving as a depository of many immunological molecules (Esteban, 2012). These protective properties have led to extensive research on the antibacterial activity of fish skin mucus (Díaz-Puertas et al., 2023;

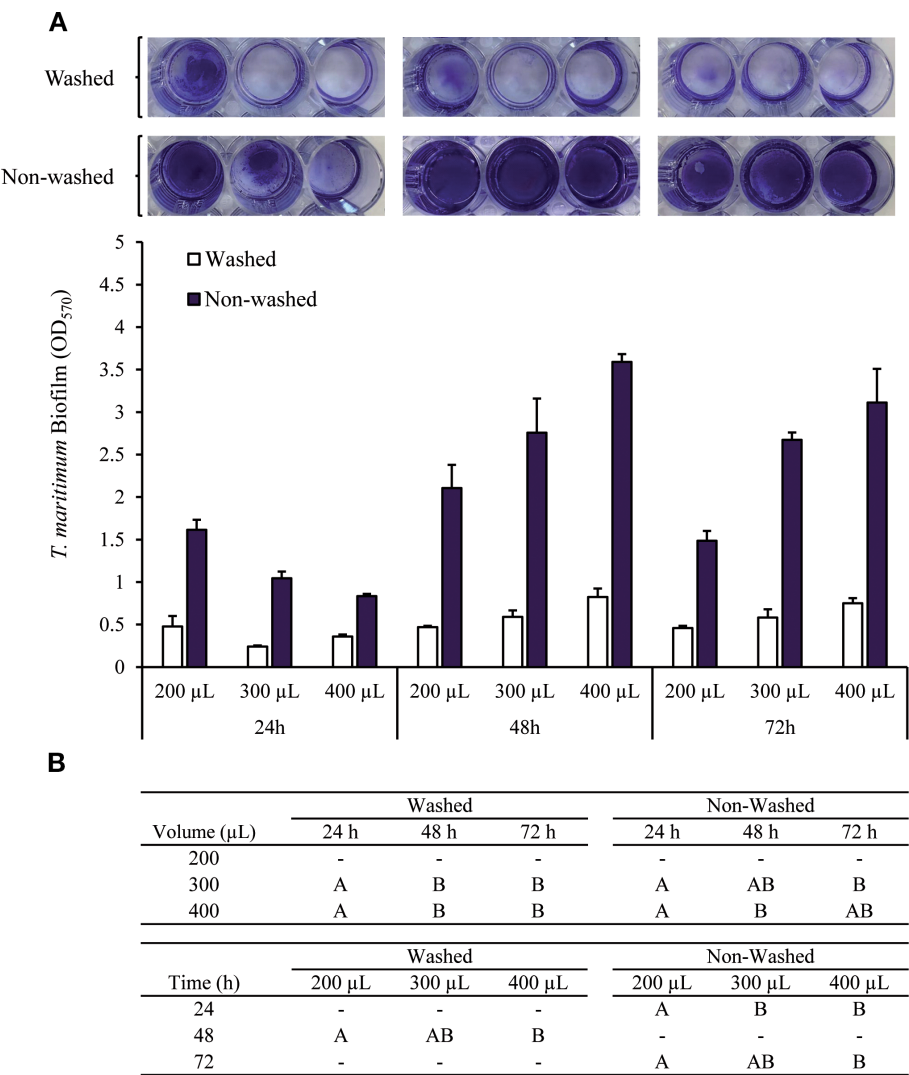


FIGURE 2 *T. maritimum* biofilm biomass determined by CV staining after 24, 48, or 72 hours of incubation at 25 °C. The values were corrected by subtracting the respective media negative control value. (A) Washing (twice) or not the microplate wells before fixing and staining the biofilm biomass, and using different volumes of bacterial suspension (200, 300, and 400 µL). (B) Capital letters indicate significant differences between bacterial suspension volumes used after the same incubation period, and differences between incubation time (one-way ANOVA, $p < 0.05$).

Tiralongo et al., 2020). The growing interest in this field is further supported by the fact that mucus can be collected using non-invasive methods (Sanahuja et al., 2023b), making it an attractive subject for study.

Different approaches to evaluate the effect of fish skin mucus on *T. maritimum* planktonic growth have been previously reported (Echeverría-Bugueño et al., 2023; Guardiola et al., 2019; Mabrok et al., 2016). The present study revealed that gilthead seabream skin mucus strongly inhibited *T. maritimum* planktonic growth (Figure 1). Our findings contradict those reported by Magariños et al. (1995), who observed resistance of several *T. maritimum* strains, including the one used in this study, to the antibacterial activity of seabream, turbot, and seabass skin mucus. The discrepancies between these results could arise from variations in methodologies employed for mucus processing and antibacterial activity testing. Firstly, the mucus processing reported by these authors did not include a homogenization and centrifugation process to remove the insoluble fraction of mucus. As a result, the soluble antimicrobial molecules could have remained trapped within the mucin matrix, thereby limiting their ability to interact with bacterial cells. Besides, before the antibacterial activity testing and probably to obtain a more homogenous sample to work with, a 1:20 dilution of the crude mucus was made in seawater. Regarding the antimicrobial testing, the referred work uses the disk diffusion method on agar plates. This method is widely established in clinical microbiology but, despite it has been reported for *T. maritimum* agar-supported antimicrobial susceptibility testing (Avenida-Herrera et al., 2005), standard protocols have not been developed for MIC or disc diffusion assays for this species (Smith, 2019). The planktonic growth inhibition assay protocol used in the present study was adapted from a previously established methodology applied to other marine pathogens (Sanahuja et al., 2019) and it has been proven to be suitable for *T. maritimum*. Other approaches to evaluate the effect of fish skin mucus on *T. maritimum* growth in a liquid medium have been previously reported using Senegalese sole skin mucus (*Solea senegalensis* Kaup) (Guardiola et al., 2019; Mabrok et al., 2016). When using diluted mucus samples in TBS, low antibacterial activity was detected (Mabrok et al., 2016), whereas when using the soluble fraction of non-diluted skin mucus, higher inhibitions (20–90%), similarly to our results, were observed (Guardiola et al., 2019). It has been reported that Atlantic salmon skin mucus exhibits a strong antibacterial activity against *T. dicentrarchi* and *T. maritimum*, even though being previously diluted (Echeverría-Bugueño et al., 2023). The antibacterial activity of skin mucus against different pathogens has been described to be fish species-dependent (Sanahuja et al., 2019), outlining the importance of standardizing protocols for each fish species. The results obtained in the present study suggests that mucus sample dilution should be avoided when specifically studying the antibacterial activity of gilthead seabream skin mucus on *T. maritimum* planktonic growth.

Bacterial biofilms constitute significant challenges in medical and ecological contexts due to their remarkable ability to resist

antibiotic treatments, leading to persistent infections (Flemming et al., 2016). Therefore, research efforts are being made to develop new strategies to control the formation of these structures, such as the phage therapy (Wroe et al., 2020), the search of new molecules derived from plant extracts (Rather et al., 2021b), or the use of nanoparticles for the delivery of antimicrobial compounds (Rather and Mandal, 2023). Biofilms have been associated with fish skin infections caused by different pathogens, such as *Vibrio anguillarum* (Croxatto et al., 2007), *Piscirickettsia salmonis* (Levipan et al., 2020), and *T. maritimum* (Nowlan et al., 2021). Unfortunately, few therapeutic options are currently available to control biofilm formation or to treat established biofilms (Koo et al., 2017). This study aimed to provide a platform for studying the potential antimicrobial effects of fish skin mucus on the formation and dispersion of biofilms of *T. maritimum*, an important pathogen in aquaculture.

The ability of *T. maritimum* to rapidly form a biofilm has been previously described (Levipan et al., 2019). Multilayered-like cell aggregates are already detected after 24 h incubation, and the biofilm biomass produced remains stable during the next four days. These results match with the profile observed in this report, as no differences were found whether cells were incubated for 24, 48, or 72 hours when using 200 µL of bacterial cultures (Figure 2). While the highest biofilm biomass was achieved using 400 µL of culture and 48–72-hour incubation periods, we used 200 µL inoculum with a 24-hour incubation time to investigate the effects of skin mucus on *Tenacibaculum maritimum* biofilm growth. This approach was chosen for two key reasons: i) it minimizes the overall experimental time and ii) it reduces the amount of fish skin mucus required for each assay, which is often a limited and valuable resource. Moreover, as microplates operate in batch mode, meaning nutrients are depleted and metabolic wastes accumulated, they are more suited for short-term experiments (Gomes and Mergulhão, 2021).

The biofilm generated under the experimental conditions used can be easily disturbed during conventional washing step prior biofilm formation. We demonstrate that eliminating the washing steps, a thick biofilm can be detected and quantified. While this step is typically used to remove planktonic and sedimented cells that are not part of the biofilm structure, it can cause unintended detachment of an unpredictable number of adherent microorganisms. This unintended detachment can lead to poor reproducibility in biofilm biomass measurements (Azeredo et al., 2017; Coenye and Nelis, 2010; Gomes and Mergulhão, 2021; Gómez-Suárez et al., 2001). Although it has been described that this pathogen strongly adheres to hydrophobic surfaces, such as polystyrene (Burchard et al., 1990; McEldowney and Fletcher, 1988; Sorongon et al., 1991), the results obtained after a two-washes step results in a high loss of *T. maritimum* biofilm biomass (Figure 2) (see Supplementary Material). Accordingly with our results, recent studies using microplates describe the ability of this bacteria to produce biofilms that were quantified by CV staining without previous washes (Escribano et al., 2023; Levipan et al., 2019).

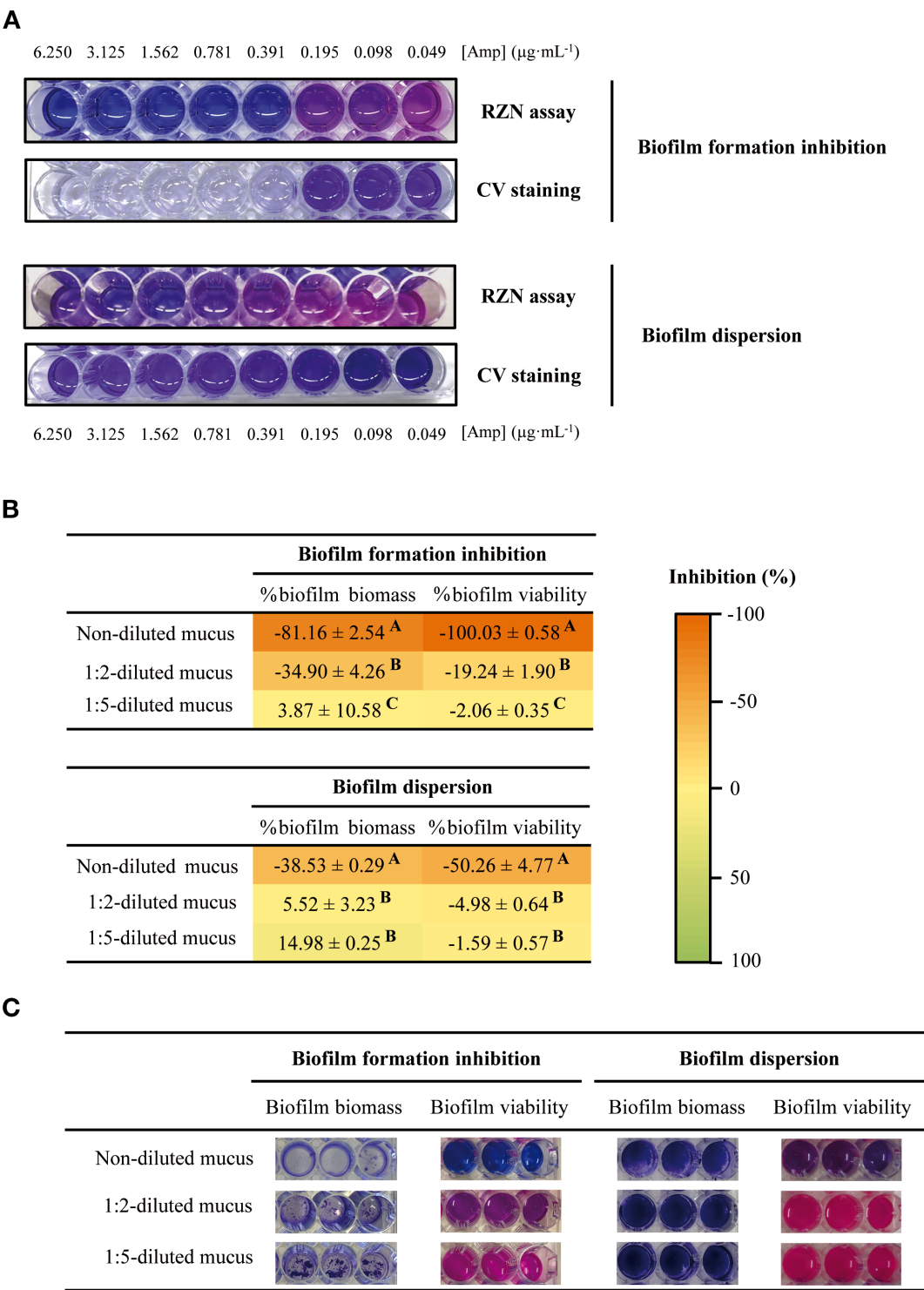


FIGURE 3
(A) Susceptibility dose-curves with 2-fold serial dilutions of ampicillin on inhibition of biofilm formation (upper panels) and dispersal of preformed biofilm (bottom panels) assays, by CV and RZN staining. (B, C) Effect of skin mucus dilutions on *T. maritimum* biofilm biomass and biofilm viability in the biofilm formation and dispersion assays. Three different concentrations of mucus were used, corresponding to 9.66, 4.83 and 1.93 mg protein/mL (non-diluted, 1:2-diluted mucus and 1:5-diluted mucus respectively). Differences between the effects of different skin mucus dilutions are indicated by capital letters (one-way ANOVA, $p < 0.05$).

Nevertheless, it cannot be ruled out a different attachment of *T. maritimum* on different types of surfaces.

To study the antibacterial properties of various compounds against *T. maritimum* biofilms, this study proposes the use of uncoated 48-well polystyrene microplates and to omit washing steps prior to crystal violet (CV) and resazurin (RZN) staining. This approach streamlines the experimental process while maintaining accuracy in biofilm assessment.

Once established the conditions for biofilm formation, the effects of gilthead seabream skin mucus in *T. maritimum* biofilm formation and dispersion have been tested. The results show that the mucus inhibited *T. maritimum* CECT 4276 biofilm formation in a concentration-dependent manner (Figure 3B). Having in consideration the results of the effect of skin mucus on planktonic growth, the inhibition of biofilm formation observed could be a consequence of the inhibition of total bacterial growth in the presence of the skin mucus, as showed in the planktonic growth assays (Figure 1).

While a commercial vaccine exists to prevent *T. maritimum* infections in turbot, the control of tenacibaculosis in other cultured species primarily relies on curative treatments, particularly antibiotics. As the infection by this pathogen is characterized by the formation of a biofilm (Nowlan et al., 2021), research into novel antimicrobial agents should focus not only the ability to inhibit the biofilm formation, but also the ability to eliminate a pre-existing biofilm.

This dual approach is crucial because *T. maritimum* rapidly forms biofilms on various surfaces, and these biofilms enhance the pathogen's environmental persistence and facilitate disease transmission. Effective control strategies must address both preventing new biofilm formation and eradicating existing biofilms to combat tenacibaculosis in aquaculture settings. Our results demonstrated that, in addition to inhibit biofilm formation, seabream skin mucus could alter previously formed *T. maritimum* biofilms, reducing both biofilm biomass and cell viability (Figure 3B). Similarly, it has been previously reported that the skin mucus of the freshwater fish *Puntius sophore* can distort the preformed biofilms as well as obstruct the adhesion property of different human bacterial pathogens (Patel et al., 2020). However, as expected, the impact of skin mucus on the disaggregation of *T. maritimum* pre-formed biofilms was found to be less pronounced than that observed in the biofilm formation assays, evidencing the high resistance that these structures confer to the bacterial cells. This limited dispersal effect represents a biological limitation of the mucus.

To our knowledge, this is the first study to provide a simple methodology for assessing the impact of skin mucus on both the formation and dispersion of *T. maritimum* biofilms.

5 Conclusions

T. maritimum infections are characterized by the formation of a biofilm on the fish skin. Hence, studies providing knowledge on its pathogenicity and possible treatments should include the formation of these structures in addition to working with its planktonic form. Here, we described a simple and rapid protocol for assessing the antimicrobial effects of fish skin mucus on *T. maritimum* biofilm growth in polystyrene microplates. The results showed that gilthead seabream mucus strongly inhibited *T. maritimum* biofilm formation, and it reduces the biomass and viability of previously formed biofilms. Therefore, the protocols employed could be used as a tool to study how different fish culture conditions affect fish susceptibility to *T. maritimum* infections. Moreover, it opens the door to investigate the components of fish skin mucus and the molecular mechanisms involved in its antibiofilm activity, with the aim of developing new strategies to control these structures.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was conducted in accordance with the Guiding Principles for Biomedical Research involving Animals (EU2010/63), the guidelines of Spanish legislation (Law 32/2007 and RD 53/2013) and approved by the Ethics Committee of the University of Barcelona for the Use of Laboratory Animals and the Generalitat de Catalunya (DAAM 9383). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MT: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. IS: Data curation, Formal analysis, Writing – review & editing. CB: Funding acquisition, Supervision, Validation, Writing – review & editing. AI: Funding acquisition, Supervision, Writing – review & editing. CM: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing. LF-A: Conceptualization, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1631980/full#supplementary-material>

SUPPLEMENTARY VIDEO 1

Video showing the washing procedure of *T. maritimum* biofilms incubated during 24h at 25°C in 48-well polystyrene plates before biofilm fixing.

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